A BIOCHEMICAL CHARACTERIZATION OF SIMIAN ROTAVIRUS SA-11

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ABSTRACT

BIOLOGY

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A Biochemical Characterization of Simian Rotavirus SA-11

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Simian rotavirus SA-11 was propogated in MA-104 (embryonic rhesus) monkey kidney cells. Infectivity of the virus was greatly enhanced by treating the innoculum with trypsin. Virus purified by equilibrium viscosity density gradient sedimentation in saturated potassium tartrate - 30% glycerol gradients banded at a density of 1.35g/ml. Viral structural polypeptides and RNA were analysed by discontinuous SDS-polyacrylamide gel electrophoresis. Eight structural polypeptides were resolved with apparent molecular weights of 3.06×10^4 to 1.13×10^5. Two of the polypeptides composed 60% of the virus polypeptides and were associated with the inner capsid of the virus. Five of the polypeptides were tentatively assigned to the inner capsid and three to the outer capsid. One or more of the polypeptides could be labeled with glucose and are therefore tentatively identified as glycopolypeptides. Eleven segments of DS-RNA could be resolved by PAGE from purified virus extracted

with phenol. They had apparent molecular weights which ranged from 1.6×10^5 to 2.41×10^6 daltons. These segments could be divided into 4 distinct size classes. Radioisotope labeled proteins and RNA were resolved on a single gel by staining and fluorography. Three additional RNA segments were resolved after analysis of dissociated whole virus (not phenol extracted). These segments probably did not represent additional RNA unresolved previously but probably represent genome RNA segments still associated with viral protein.

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CHAPTER I

INTRODUCTION

The genus Rotavirus is a member of the Reoviridae family of viruses. The genus consists of numerous morphologically indistinguishable and antigenetically related viruses which cause enteritis primarily in young mammals and Aves. An update of rotavirus taxonomy from the Fourth International Congress of Virology, 1978 is presented in Intervirology, 11:133-135, 1979.

The first report on what is now known as rotavirus enteritis was made by Light and Hodes (1943, 1949). These workers isolated a filterable agent from the stool of young children with enteritis and showed that the isolate caused diarrhea in newborn calves. Hodes (1977) has since shown that the morphology of this early isolate was identical to later rotavirus isolates. Since the reports of Light and Hodes, others have isolated viruses which had some characteristics of viruses of the Reoviridae and were later shown to be rotaviruses. Kraft (1957), Malherbe and Strickland-Cholmley (1967) and Mebus et al. (1969) reported finding such viruses in mice, monkeys and calves, respectively.

Human rotavirus was first detected in duodenal biopsies of ill infants by Bishop et al. (1973) in Australia. Since then numerous reports of human rotavirus isolation (Bishop et al. (1974), Davidson et al. (1975), Kapikian et al. (1974),

Middleton et al. (1974) have been presented. It is now known that rotaviruses are a major cause of enteritis among young children worldwide. (Editorial, The Lancet, 1975). These findings spurred intense research on this group of viruses during the 1970's.

Rotavirus has been isolated from fecal extracts of mice (Kraft, 1957), monkeys (Malherbe and Strickland-Cholmley, 1967), humans (Bishop et al., 1973, Flewett et al., 1973), foals (Flewett et al., 1975), sheep (McNulty et al., 1976, Snodgrass et al., 1976), goats (Scott et al., 1978), rabbits (Bryden et al., 1976), deer (Tzipori et al., 1976, Snodgrass et al., 1976), pronghorn antelope , apes (Ashley et al., 1975), impala, Thompson's gazelles and addox (Eugster et al., 1979), turkeys (Bergeland et al., 1978), guinea pigs and bears (Mebus, 1977), and laying hens (Jones et al., 1979). It has recently been shown that rotaviruses from Aves and mammals are antigenetically related (McNulty et al., 1979). However, most of these isolated rotaviruses have not been adequately characterized because they replicate poorly if at all in cell culture. It is not known if each of the isolates represents type species of rotavirus or if each was derived from a heterotypic infection.

It is the intent of this study to characterize the structural polypeptides and the RNA genome of simian rotavirus SA-11.

CHAPTER II

LITERATURE REVIEW

Morphology and Morphogenesis

Rotaviruses from various mammalian species are morphologically indistinguishable from one another. They have a double-shelled capsid consisting of outer and inner layers. Both double and single-shelled particles have been observed (Bridger and Woode, 1976). Much and Zajac (1972) showed that murine rotavirus was similar in morphology to single-shelled orbivirus and bluetongue virus particles. Flewett et al. (1973) showed that rotaviruses could be distinguished from reoviruses and orbiviruses by their sharply defined outer layer. Palmer et al. (1977) have estimated that complete virions are 67-68 nm in diameter and that the icosahedral core measures 38 nm in diameter. According to Martin et al. (1975) the capsomeres of single-shelled particles follow the structure of T = 9. They estimated that there were 540 structural units composing single-shelled particles. These structural units were clustered into 180 wedge-shaped trimer subunits conforming to a T = 3 morphology. At higher magnification the 32 large units were shown to be composed of trimeric wedge-shaped components which form the large surface pattern by sharing. This sharing of subunits is common to rotavirus and orbivirus single-shelled particles.

Esparza and Gill (1978) proposed a model having a T number

of 16. In this model five holes exist on each edge of the virus icosahedral. There were 162 total holes and 320 subunits. These subunits were reported as being trimeric, and gave rise to a virion with 920 protomers. Kogasaka et al. (1979) proposed a model with a capsomere number of 42 and a T number of 4.

Roseto et al. (1979) used a freeze-drying technique to study single and double-shelled particles of human, bovine and similar rotaviruses. They reported that the inner capsid was composed of 132 capsomeres having a skewed and icosahedral pattern. The pattern observed was that of T = 13. The double-shelled particles had a smooth surface which was perforated with holes. By using the freeze fracture technique Roseto et al. (1979) have shown that the holes of the outer layer correspond one to one with the holes of the inner capsid. These holes were 3 nm in diameter and were regularly organized around five and six fold axes.

Free-lying lattice sheets have been observed in some rotavirus preparations. Esparza and Gill (1978) believe that these may simply be fragments produced during specimen preparation. Holmes et al. (1975) have reported observing tubular forms of rotavirus in tissue cultures and fecal materials. At present, the significance of tubular forms of rotavirus is unknown. However, both lattice sheets and tubular forms are composed of subunits arranged in a hexagonal pattern.

McNulty et al. (1976) reported that calf rotavirus could

be observed in the rough endoplasmic reticulum and in viroplasm near the rough endoplasmic reticulum. Mature virus particles were released from the cell by passing through breaks in the plasma membrane and by a budding process (McNulty, 1976).

Buoyant Density and Sedimentation

Bridger and Woode (1977) reported that calf rotavirus double-shelled particles have a buoyant density of 1.36g/ml in CsCl and single-shelled particles have a buoyant density of 1.38g/ml in CsCl. Elias (1977) reported the same results using human rotavirus. Rodger et al. (1977) have confirmed these results using rotavirus SA-11 particles. However, Kalica et al. (1979) have determined that double-shelled SA-11 particles have a buoyant density of 1.37g/ml in CsCl while single-shelled particles have a buoyant density of 1.39g/ml in CsCl. Elias (1977) has shown that particles of density 1.36g/ml had a more than 3 $\log_{10}$ higher infectivity than those of 1.38g/ml.

No sedimentation valves for SA-11 have been reported. However, Newman et al. (1975) reported a relative S value of 500S for calf rotavirus. Petric et al. (1975) reported the sedimentation value for Infantile Gastroenteritis Virus (IGV) as 520-530S.

Stability and Infection

SA-11 is stable to freeze-thawing, sonication, incubation at 25 C overnight and at 37 C for 1 hour. The virus is not inactivated by ether, chloroform and Genetron (Estes et al., 1979).

Rodger et al. (1977) reported that SA-11 is degraded to amorphous protein at p^H3-4 in Walpole's acetate-phosphate buffer and Gomori's succinate. These findings are similar to those of Malherbe and Strickland-Cholmley (1967) who reported that p^H3 inactivates SA-11 rotavirus. Estes et al. (1979) have shown that the virus is inactivated above p^H 10.0 and by heating in 2M-$MgCl_2$ and $CaCl_2$ at 50 C. It is stabilized by heating in 2M-$MgSO_4$. There was no morphological alteration of the virus when treated with bromelain, papain, pronase, trypsin or alpha chymotrypsin (Rodger et al., 1977). However, when treated with alpha chymotrypsin in the presence of Cs^+ the virus was uncoated such that it was indistinguishable from single-shelled particles (Rodger et al., 1977). Matsuno and Mukoyama (1979) showed electrophoretically that trypsin treatment diminishes the intensity of all structural viral proteins of Nebraska Calf Diarrhea Virus (bovine rotavirus). Holmes et al. (1976) suggested that lactase present in the brush border of intestinal epithelial cells acts as a combined receptor and uncoating enzyme. However, results from studies by Rodger et al. (1977) showed that the morphology of simian and human rotavirus was not altered in the presence of beta galactosidase while calf rotavirus was uncoated by both lactase and beta galactosidase. Treatment of the virus with chelating agents such as EDTA and EGTA significantly reduces infectivity (Estes et al., 1979). Cohen et al. (1979) has shown that calcium ions are necessary for maintenance of rotavirus integrity

near neutrality. Although he was unable to determine the minimal concentration of free calcium ions required for rotavirus integrity, it was determined to be below 10μM. These findings support the hypothesis of Durham (1977), that calcium ions possibly control virus disassembly.

Virus Proteins

There is a wide variation in the number and size of reported rotavirus proteins. Rodger et al. (1975) used a discontinuous polyacrylamide gel electrophoresis (PAGE) system and found 8 polypeptides associated with human rotavirus and possibly 9 calf rotavirus polypeptides. Newman et al. (1975) used a continuous phosphate buffered polyacrylamide gel electrophoresis (PAGE) system and found that calf rotavirus was composed of 5 polypeptides. Bridger and Woode (1976) using a similar system reported finding 5 polypeptides. Todd and McNulty (1977) used a discontinuous tris-glycine buffered technique described by Laemmli (1970) and were able to resolve 10 polypeptides for lamb rotavirus. Obijeski et al. (1977) used both continuous and discontinuous systems and resolved 10 structural proteins for human rotavirus. Rodger et al. (1977) have resolved 8 or 9 polypeptides for human rotavirus. They have also performed the most detailed investigation of the simian rotavirus, SA-11. Using a discontinuous system as described by Laemmli (1970) they resolved 9 SA-11 polypeptides. However, one of these, a 58,000 component was not always present. The molecular weights of these polypeptides

ranged from 1.33×10^5 to 14×10^4 daltons. Six of these polypeptides were associated with the inner shell. There were three major proteins resolved with the remaining being minor proteins. Of the outer shell components one is glycosylated (by PAS stain) and one is the major outer shell component (Rodger et al., 1977). Kalica and Theodore (1979) have reported that simian rotavirus, SA-11, has eight structural proteins. Five of the viral proteins were listed as inner shell components and the remaining components were associated with the outer shell. They suggest that one or all of the outer shell polypeptides are associated with a hemaglutinin (HA).

Cohen et al. (1978) used Conconavalin A to show that the outer capsid of calf rotavirus was glycosylated. He proposed that the terminal carbohydrate residue of the glycoprotein was mannose. Cohen (1977) showed that rotavirus possessed an RNA polymerase. More recently he has determined that this polymerase is associated with the single-shelled particles (Cohen et al., 1979). He reports the detection of two glycosylated outer coat polypeptides in the low molecular weight range (3.4 and 3.1×10^4 daltons).

Thouless (1979) used a polyacrylamide gel system described by Spear and Roizman (1972) and resolved eight structural polypeptides for calf, pig, lamb, mouse, foal, rabbit and human rotaviruses. She also resolved three nonstructural polypeptides in pig, lamb and mouse rotavirus and two nonstructural polypeptides in calf, foal, rabbit and human rotavirus. She reported

that rotavirus polypeptides begin appearing four to five hours post-infection and that synthesis of nascent viral protein is maximal at eight hours post infection. Viral polypeptides were still being produced fifteen to eighteen hours post-infection. McNulty et al. (1976) observed calf rotavirus by EM and reported that even though there was a distension of cisternae of the rough endoplasmic reticulum (RER) fourteen hours post-infection, very few particles could be visualized. However, by twenty-two hours post-infection there were many virus particles observed within distended RER and matrices of viroplasm could be observed near developing virus.

Thouless (1979) found four proteins associated with the inner coat of calf rotavirus and four proteins associated with the outer coat. Several of the outer coat polypeptides altered molecular weights on maturation indicative of post-syntheses processing. Matsuno and Mukoyama (1979) used a discontinuous system and pulse labelling to resolve eight structural polypeptdes of calf rotavirus. They also detected three nonstructural proteins. Five of the structural proteins were listed as inner shell components and three were outer shell components. One of the outer shell peptides was shown to be glycosylated by ^{3}H glucosamine labelling. They found that in infected cells infectious virus was produced as early as four hours post-infection, increased exponentially and peaked at ten to twelve hours post-infection. They reported that there was no post-synthesis modification of any viral

polypeptides and therefore all of the polypeptides represent primary gene products.

SA-11 RNA

Welch (1971) performed the initial nucleic acid characterization of rotavirus and showed that the viral genome of calf rotavirus consisted of RNA. Much and Zajac (1972) confirmed this finding. Subsequent investigations by Welch and Thompson (1973) showed that the genome was double-stranded RNA. Newman et al. (1975) and Rodger et al. (1975) determined that calf rotavirus was not only double stranded but also segmented having eleven to twelve segments. Newman et al. (1975) proposed a coding relationship between RNA and protein. The total molecular weight of the viral genome was 1.1 to 1.2 x 10^6 with a range of 2.0 x 10^5 to 2.2 x 10^6. Later reports on human rotavirus by Kalica et al. (1976) and Schnagl and Holmes (1976) gave similar results. Todd and McNulty (1976, 1977) reported finding eleven to twelve RNA segments in pigs and lambs. Kalica et al. (1976) found that both SA-11 and the "O" agent had eleven to twelve segments. Obijeski et al. (1977) reported that human rotavirus possessed a genome of fifteen segments of RNA.

There are variations in sizes of the RNA segments between human isolates (Schnagl and Holmes, 1976). Verly and Cohen (1977) reported finding size variations in RNA segments from different calf rotavirus isolates. Kalica et al. (1978) compared human and several animal rotavirus RNA's finding that

the number of interspecies differences was greater than the number of intraspecies differences.

Cohen (1977) detected the presence of a ribonucleic acid polymerase associated with calf rotavirus. He has since determined that this enzyme is associated with single-shelled particles and may be activated by calcium chelation. Matsuno and Mukoyama (1979) suggest that most if not all of the RNA segments of rotavirus are probably monosystronic messages.

CHAPTER III

MATERIALS AND METHODS

Cell Culture and Virus Growth

The MA-104 line of embryonic rhesus monkey kidney fibroblast cells (Microbiological Associates, Walkersville, Md.) was grown in 490 cm^2 (Corning Glass Works, Corning, N.Y.) plastic bottles. Cells were grown to confluency using Eagle's minimal essential medium (MEM; Eagle, 1959) containing 10% fetal bovine serum. After infection, confluent monolayers were maintained on MEM with 10% Earle's salts without fetal bovine serum. Roller bottles were seeded and cultured at 37 C.

Mouse L 929 cells were grown to confluency the same as MA-104 cells. However, after infection monolayers were maintained on MEM with 10% Earle's salts and 2% fetal bovine serum.

Virus Source and Propogation

Simian rotavirus SA-11 was obtained from Dr. G. William Gary, Jr., reovirus type 3 from Dr. Erskine Palmer and purified dissociated vesicular stomatitis virus from Helen Lindsey-Regnery, Center for Disease Control, Atlanta, Ga.

Upon confluency monolayers were washed twice with Hanks' Basic Salt Solution with 0.5% $NaCO_3$, p^H 7.2-7.4. MA-104 monolayers were infected with 15 ml (enough virus to insure an input multiplicity of 10-20 particles per cell) per bottle of SA-11 MA-104 cell suspension. Prior to infection virus-cell

suspension was incubated for 1 hr at 37 C in the presence of 10 μg/ml of trypsin (Difco Laboratories, Detroit, Mich.). Mouse L cells were innoculated with 2 ml (10-20 particles per cell) per bottle of Reovirus type 3. All virus was allowed to adsorb for 1 hr at 37 C with slow rolling. Cultures were incubated until approximately 90% of the monolayer displayed characteristic cytopathic effect (CPE).

Virus proteins were radiolabeled with radioisotopes, the infected monolayer cultures of MA-104 cells were incubated in 490 cm^2 roller bottles with MEM containing one-tenth the normal concentration of amino acids and either 3.9 μCi ^{3}H amino acids/ml media or 0.66 μCi ^{14}C leucine/ml media. Nucleic acids were labeled with 3 μCi ^{3}H uridine/ml of media. Glucosamine uptake was detected by omitting glucose from MEM and labeling the virus with 0.066 μCi ^{14}C glucosamine/ml of media.

Virus Harvest and Purification

Virus was harvested by collecting culture media in 125 ml conical, glass, graduated centrifuge bottles (Bellco Glass Co., Vineland, N.J.). Cells adhering to culture bottles were scrapped from the bottle surface using a rubber policeman. These cells with associated virus were removed from culture bottles by washing with TCN buffer, p^H 7.2-7.4 (10 mM Tris-HCl, Sigma Chemical Co., St. Louis, Mo., 10 mM $CaCl_2$, Fisher Scientific Co., Fair Lawn, N.J., 100 mM NaCl, Sigma). These washings were collected in conical glass graduated centrifuge bottles (Bellco). Virus infected cells were centrifuged in a

PR-2 International Centrifuge (International Equipment Co., Needham Hts., Mass.) at 5 C. The supernatant was discarded and the cell pellets were resuspended using TCN buffer. Cell pellets were combined with one half volume of Genesolv D (Allied Chemical Co., Morristown, N.J.) in conical plastic 50 ml centrifuge tubes (Corning) and vigorously vortexed for 3-5 min. The aqueous phases were separated by centrifuging for 10 min at high speed in an IEC Clinical Centrifuge (International Equipment Co.). The aqueous phases were removed and pooled. Ten ml of TCN buffer was added to the interphase and Genesolv D. The suspension was vortexed 3-5 min vigorously and centrifuged as above. This extraction procedure was repeated twice. The pooled aqueous phases were reextracted with one fourth volume of Genesolv D, vortexed 2 min and centrifuged for 5 min at high speed in an IEC clinical centrifuge.

Virus was concentrated from aqueous phases by centrifugation at 35,000 RPM for 2 hr in a Beckman SW41 rotor at 5 C in a Beckman Model L2-75B Ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). The supernatant was removed, discarded and the virus pellet resuspended in TCN buffer. The suspension was layered onto preformed 30% glycerol (Fischer) saturated potassium tartrate (Fischer) (Gly-KT) equilibrium viscosity density gradients (in TCN buffer) and centrifuged for 16 hr at 35,000 RPM in a Beckman SW40 rotor. Reovirus type 3 was harvested and purified by the same procedure. Gradients were fractionated and 0.5 ml fractions were collected from the bottom of the tube.

Radioactivity was detected by counting a 0.005 ml portion of each fraction in a Bioflour scintillation counting cocktail (New England Nuclear, Boston, Mass.). Radioactivity was measured using a Searle Mark III 6880 Liquid Scintillation System.

Density Determinations

Estimations of density were done by weighing 100 µl samples from gradient fractions on a Mettler analytical balance.

Electron Microscopy

Virus was quantitated by electron microscopic examination. Samples from virus bands were prepared for electron microscopy by the pseudoreplica technique as described by Palmer et al. (1975) and illustrated in Fig. 1. Briefly, a drop of virus sample was placed on a block of 2% agar (about 2 cm^2). The sample was permitted to air dry. Formvar dichloride (Ernest F. Fullam, Inc., Schenectady, N.Y.) was pipetted onto the adsorbed virus and allowed to dry. Excess formvar was removed by chopping away the edges of the agar block. The preparation was stained by floating the sheet of formvar off the agar block onto a 0.5% uranyl acetate (Fischer) solution, p^H 4.5. A copper grid was placed onto the formvar sheet. This grid was then lifted from the staining solution using a small metal bar and was blotted dry with filter paper. Grids were examined using a Phillips 201 electron microscope.

Preparation of Protein and RNA Extraction

Virus determined to be free of cell debris by electron microscopy was dialysed against distilled water for 16 hr at

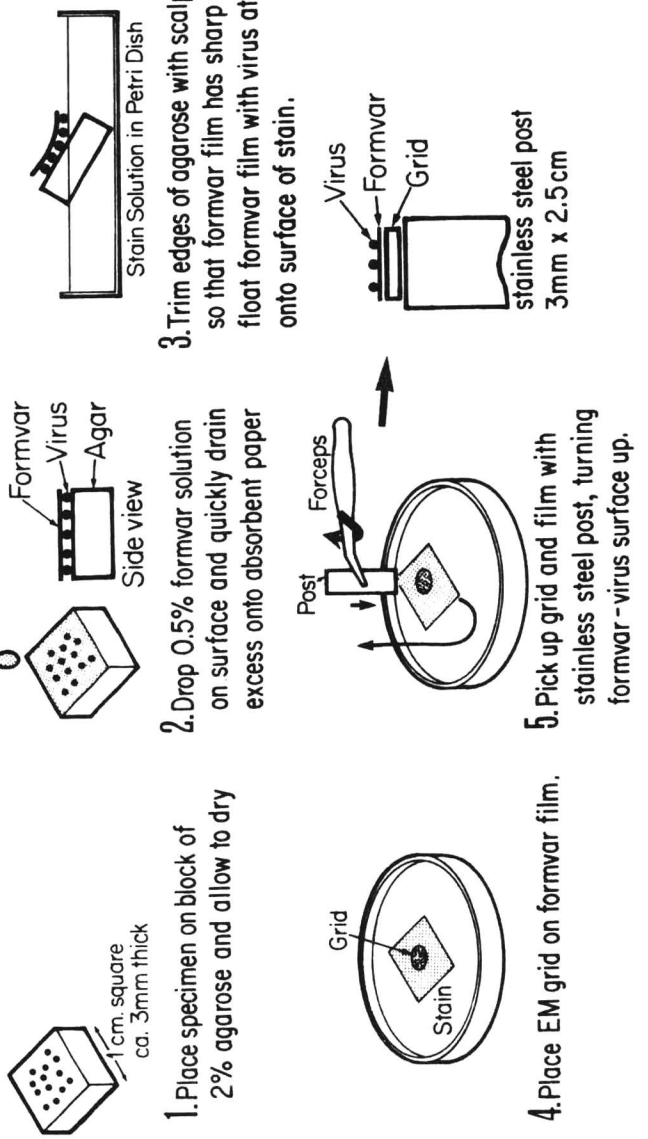

Fig. 1. Diagram illustrating the pseudoreplica technique for negative stain electron microscopy (described in Materials and Methods).

4 C and concentrated by centrifugation at 35,000 RPM for 2 hr in a spinco SW41 rotor. For protein analysis pelleted virus was disrupted with 100 μl disc dissociation buffer (DDB) (5% SDS, Gallard-Schlesenger Chem. Mfg. Corp. Carle Pl., N.Y., 10% 2 Mercaptoethanol, BDH Chemicals, Poole, England, 20% glycerol, Fischer, and 0.0625 M Tris-HCl, Sigma) and by heating in boiling water for 1 min. Ten μl of 0.1% bromphenol blue (BioRad Laboratories, Richmond, Calif.) tracking dye was then added to the dissociated virus sample.

RNA Extraction

Purified virus was resuspended in 1.0 ml TSE buffer (20 mM Tris-HCl, p^H 7.5, Sigma, 150 mM NaCl, Fischer, 2 mM disodium EDTA, Sigma) and sonicated for 1 min to disperse the virus particles. Ten μl of 2 ME and 0.1 ml of 10% SDS were added to the virus and an equal volume of phenol mixture (100 mg phenol, Bethesda Research Laboratory, Bethesda, Md., 48% chloroform, Fischer, 2% isoamyl alcohol, Fischer, and 0.1% 8 hydroxyquinoline, BDH Chemicals, England) was added to the preparation and vortexed for 3-4 min. Phenol and aqueous phases were separated by centrifuging at 10,000 RPM for 5 min at 25 C in a J-21C centrifuge. The phenol phase was removed and an equal volume of phenol mixture was added to the interphase and aqueous phase and the mixture treated as previously described. This extraction was repeated twice. The viral nucleic acids were precipitated from the aqueous phase with 2 volumes of chilled ethyl alcohol in the presence of 0.2 M LiCl (Fischer)

and stored at -20 C. RNA was removed by centrifuging at 10,000 RPM for 30 min at 4 C in a J-21C centrifuge. Alcohol was decanted from the pelleted nucleic acids and the sample was allowed to air dry. Pelleted nucleic acids were covered with 100 μl of TPE (0.003M Tris-Base, Sigma, 0.003M $NaH_2PO_4 \cdot H_2O$, Sigma, 0.0001M disodium EDTA, Sigma, 1% SDS Gallard Schlesenger, 10% glycerol, Fischer) buffer. A 15 μl aliquot of the nucleic acid samples was removed and combined with 1 ml of distilled water. Absorbancy of the sample was measured at wavelengths from 220 nm to 320 nm using a Beckman model 25 spectrophotometer to determine purity of sample. Nucleic acid samples were dialysed for 72 hr against 25 mM Tris-HCl and 1 mM disodium EDTA at 4 C. The purity of the sample was once again checked as previously described. Nucleic acids were precipitated with 2 volumes of chilled ethyl alcohol in the presence of 0.2M LiCl and stored at -21 C. The precipitate was collected as described previously. Alcohol was decanted and the sample allowed to air dry. Pelleted nucleic acids were covered with 100 μl of disc dissociation buffer. Ten μl of tracking dye (75% glycerol, BDH Chemicals, 0.1% bromcresol green, BioRad, 0.1% xylene cyanole FF, BioRad, 0.1M Tris-HCl) was added to the samples.

Reovirus type 3 proteins and RNA were isolated the same way.

Polyacrylamide Gel Electrophoresis

Virus proteins were separated by discontinuous (disc) SDS polyacrylamide gel electrophoresis prepared by the method of

Laemmli (1970). Discontinuous gels of 8% (resolving) or 10% (resolving) and 3% (stacking) gels were made from stock solution of acrylamide 60% (BioRad) and 1.6% N,N Methylenebis-acrylamide (Bis) in distilled water. The resolving gel consisted of: 8% or 10% acrylamide, 0.2% or 0.27% Bis, 0.1% SDS and 0.375M Tris-HCL p^H 8.8. The stacking gel was as follows: 3% acrylamide, 0.08% Bis, .125M Tris-HCl p^H 6.8 and 0.1% SDS. Polymerization of both stacking and resolving gels was catalyzed with final concentrations of 0.15% N,N,N,N tetramethylenediamine (TEMED) (BioRad) and 0.05% ammonium persulfate (AP) (Sigma).

Both cylindrical and slab gels were used. For cylindrical gels support tubes, 0.5 cm X 15 cm were filled 4/5 capacity, with 2.2 ml of 10% acrylamide perparation and surfaces were immediately overlayed with 0.4 ml of distilled water. The resolving gel was polymerized at room temperature for 45 min. After polymerization the water overlay was carefully removed and 0.4 ml of the stacking acrylamide preparation was layered on top of the resolving gels. The stacking gel was immediately overlayed with distilled water and allowed to polymerize for 45 min at room temperature. The gels were inserted into a Buchler upright electrophoresis chamber equipped with upper and lower reservoirs for buffer. The electrode buffer was p^H 8.3 and was composed of 0.025M Tris-base, 0.192M glycine (Sigma) and 0.1% SDS.

Slab gel components were prepared in the same manner as described for cylindrical gels. 19cm x 15.9cm glass plates separated by 1.0 mm thick spacers were filled 3/4 capacity with 30 ml of 10% or 8% acrylamide preparation and immediately layered with 1 ml distilled water. Resolving gels were allowed to polymerize for 45 min at room temperature. After polymerization distilled water was removed and 7 ml of the 3% acrylamide was layered onto the resolving gel. Immediately after layering of the stacking gel a 10 well comb was placed into the top of the stacking gel. The stacking gel was polymerized at room temperature within 45 min. Slab gels were placed on an Aquebogue upright slab gel electrophoresis apparatus equipped with upper and lower buffer chambers. The electrophoresis buffer was prepared in the same manner as described for the cylindrical gel set up except the upper electrode buffer solution contained 0.5μg/ml of Ethidium bromide (EtBr) (BioRad).

Virus RNA was separated by disc SDS polyacrylamide gel electrophoresis prepared as described previously. In some experiments gels with a 7.5% resolving gel were used. They were prepared as described above. The electrode buffer was prepared the same way as described here previously for protein slab gels.

For protein analysis a sample volume of 40μl of SA-11 rotavirus was applied to cylindrical gels. For analysis of both virus protein and RNA using slab gels sample volumes

having 3013 cpm ^{14}C leucine with 4619 cpm ^{3}H uridine was applied to gels. Sample volumes having 10, 261 cpm ^{3}H amino acids was 1652 cpm ^{14}C glucosamine was also applied to gels. Purified vesicular stomatitus virus was dissociated with DDB and co-run as a standard. 15μl of the following molecular weight standards (from Sigma) were applied to gels: bovine albumin cross linked, hemaglobin (bovine) cross linked, ad Dalton Mark VI. 40μl of Reovirus (dissociated) was applied to gels in order to determine the molecular weights of viral proteins. Gels were pre-electrophoresed for 1 hr at a constant current of 4mA prior to application of samples. Samples were electrophoresed at a constant current of 4mA/gel for 22 hr. After electrophoresis gels were stained for protein for 45 min with a staining solution composed of 0.4% Coomassie brilliant blue (BioRad) and 50% methanol (Fischer) in distilled water. Gels were destained with 7% glacial acetic acid (Fischer) and 4% methanol in distilled water. For co-electrophoresis 8μg of SA-11 and 10μg of Reo 3 DS-RNA was applied to gels. RNA gels were stained with EtBr which was included in the electrode buffer. After electrophoresis RNA gels were photographed under UV light using a Polaroid MP3 multipurpose Industrial View Land Camera and an orange filter. Destained protein gels were photographed using the same camera. Gels stained with Coomassie brilliant blue were scanned at 640 nm using a Beckman R112 gel scanner.

Molecular Weight Determinations

Molecular weight determinations of separated polypeptides and RNA were performed by PAGE as described by Shapiro et al. (1967) and Peacock and Dingman (1968). A semilog plot of known molecular weight was made of protein standards against the distance mirgrated. This same procedure was followed in determining RNA molecular weights. The protein weight standards mentioned here previously were solubilized in DDB. Double stranded Reovirus type 3 RNA was used as the RNA molecular weight standard.

Fluorography

After destaining and photography labeled gels were impregnated with EN^{3}HANCE (New England Nuclear) for 1 hr with constant gentle agitation. After impregnation the gel was placed in distilled water for 1 hr and then placed on a sheet of Whatman chromatographic paper (Schliecher and Schuell, Inc., Keene, N.J.) and dried under heat and vacuum on a BioRad slab gel dryer. The dried gel was used to expose Cronax R-4 x-ray film (DuPont Southeast, Atlanta, Ga.) with the aid of an intensifying screen (Hi Plus Cronax Medical x-ray screen, E. I. duPont de Nemours and Co., Inc., Wilmington, Del.) used to amplify fluorescence. Film was exposed at -70 C in a Kodak x-omatic cassette. After 4 days film was examined and photographed.

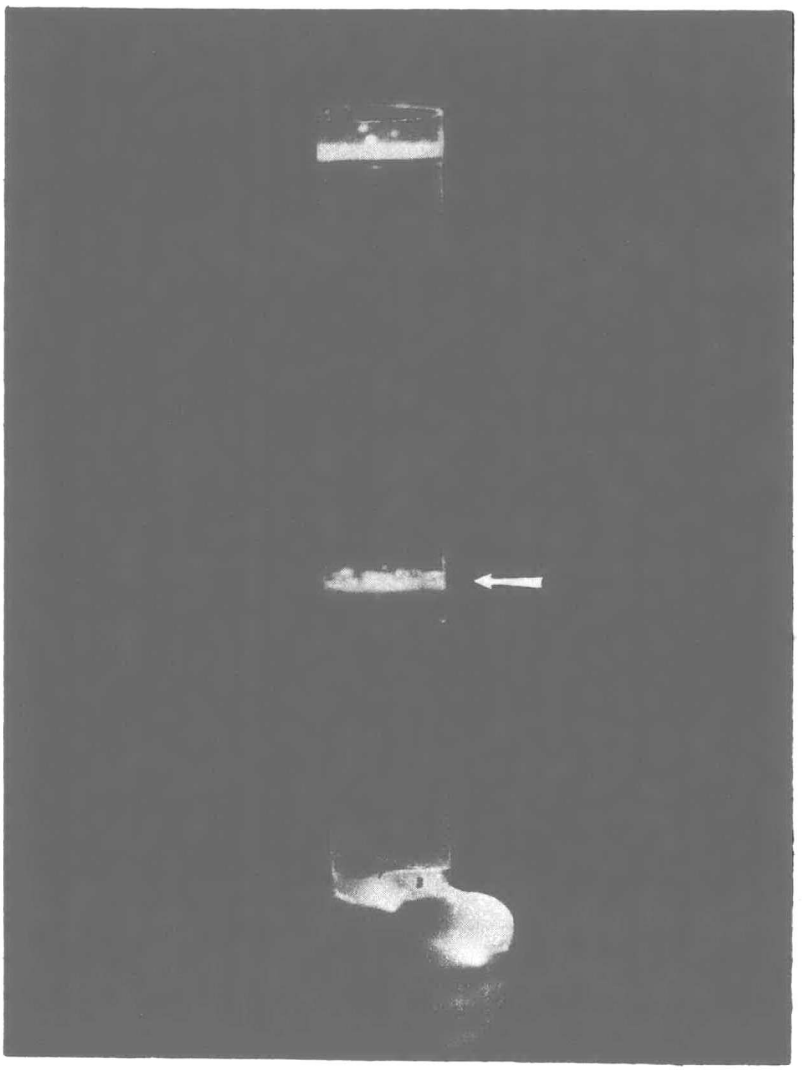

Fig. 2 Photograph of a saturated potassium-30% glycerol (KT-Glycerol) equilibrium viscosity density gradient after centrifugation of SA-11 pellet. Band (arrow) contained complete, double-shelled particles.

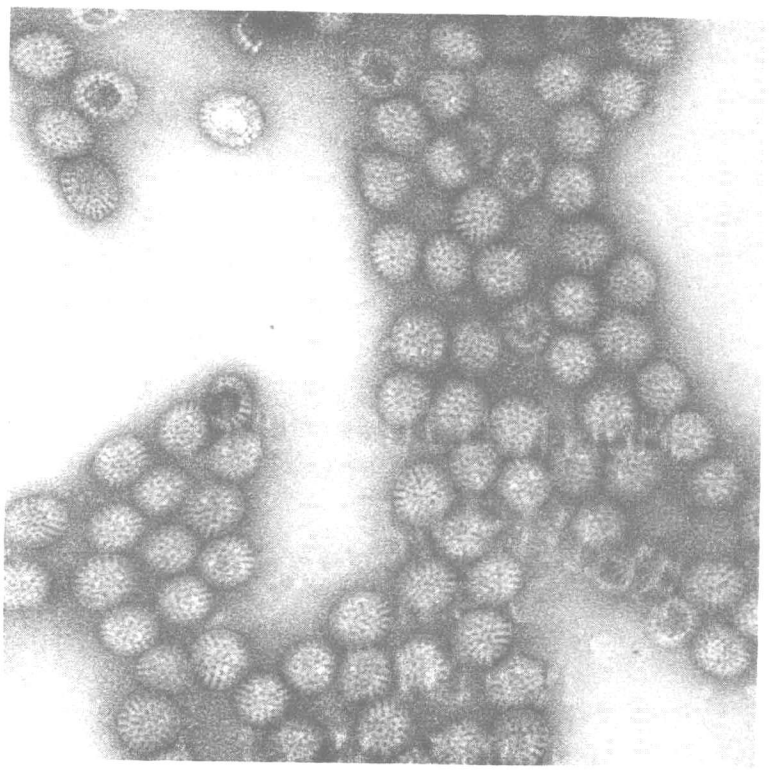

Fig. 3. Electron micrograph of particles present in virus band of SA-11 rotavirus KT-Glycerol gradient. Stained with UA. Bar denotes 100nm.

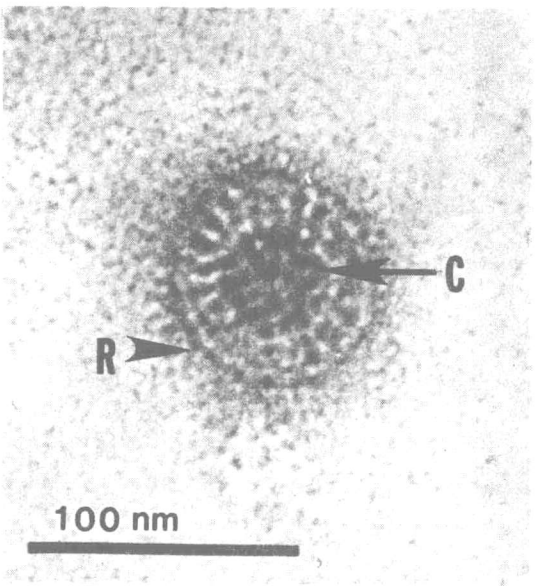

Fig. 4. Electron micrograph of complete SA-11 rotavirus particle showing well defined outer capsid (R) and core (C) which has been penetrated by UA stain. Note characteristic spoke pattern of capsomeres (arrow). Bar denotes 100 nm.

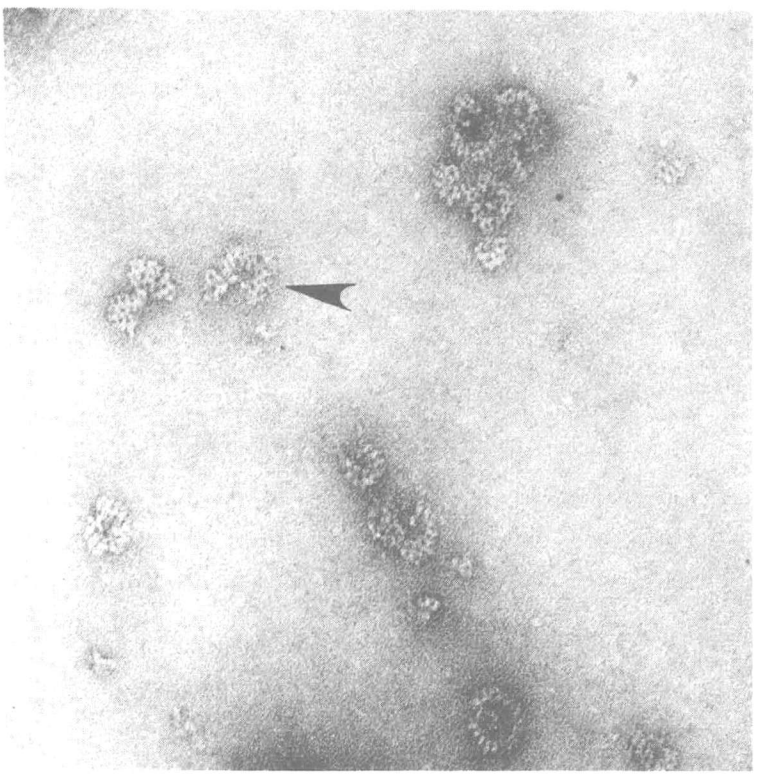

Fig. 5. Electron micrograph of disrupted rotavirus particles. Free-lying capsomeres (arrow) are evident.

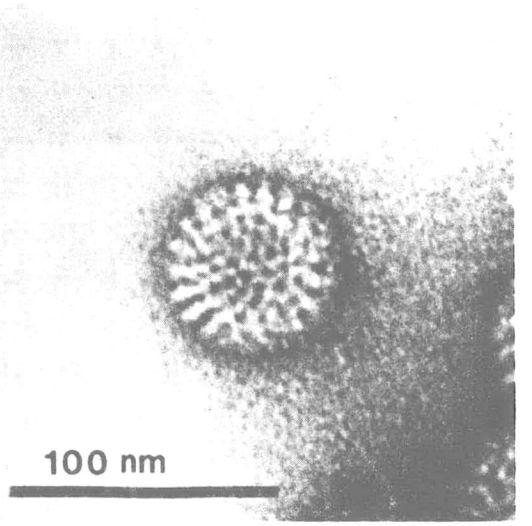

Fig. 6. Electron micrograph of single-shelled SA-11 rotavirus particle. Bar denotes 100nm.

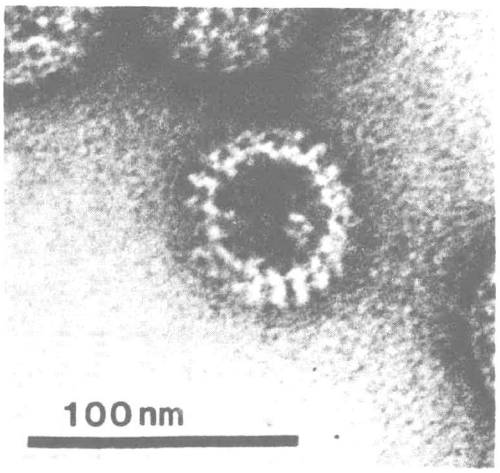

Fig. 7. Electron micrograph of SA-11 rotavirus particle in which empty capsid has been penetrated by UA stain. Bar denotes 100 nm.

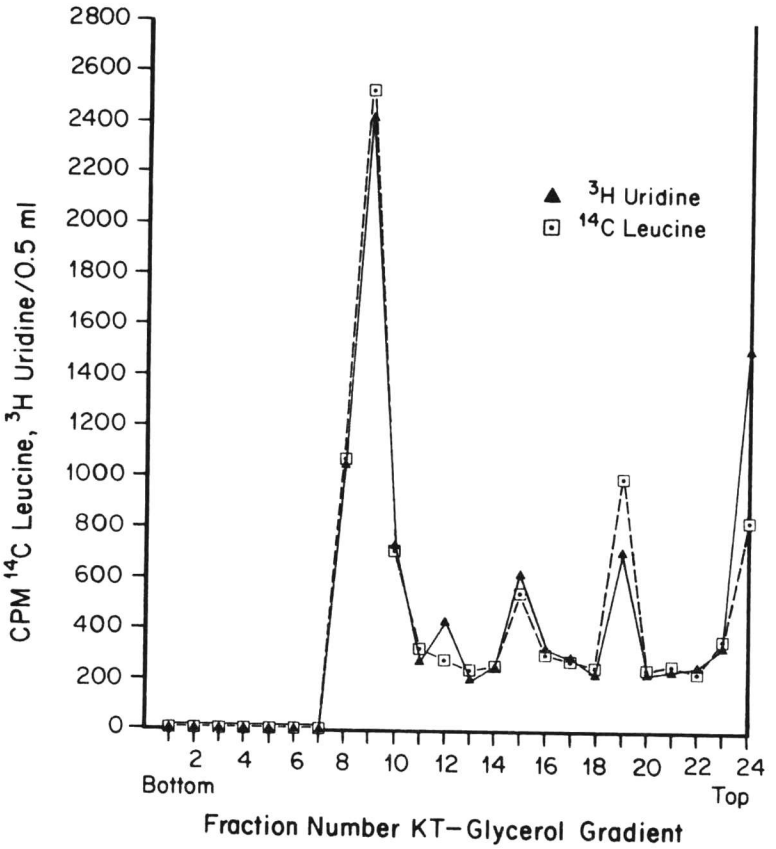

Fig. 8. Graph showing sedimentation of SA-11 rotavirus in KT-Glycerol equilibrium density gradient. Virus was labeled with ^{3}H uridine and ^{14}C leucine.

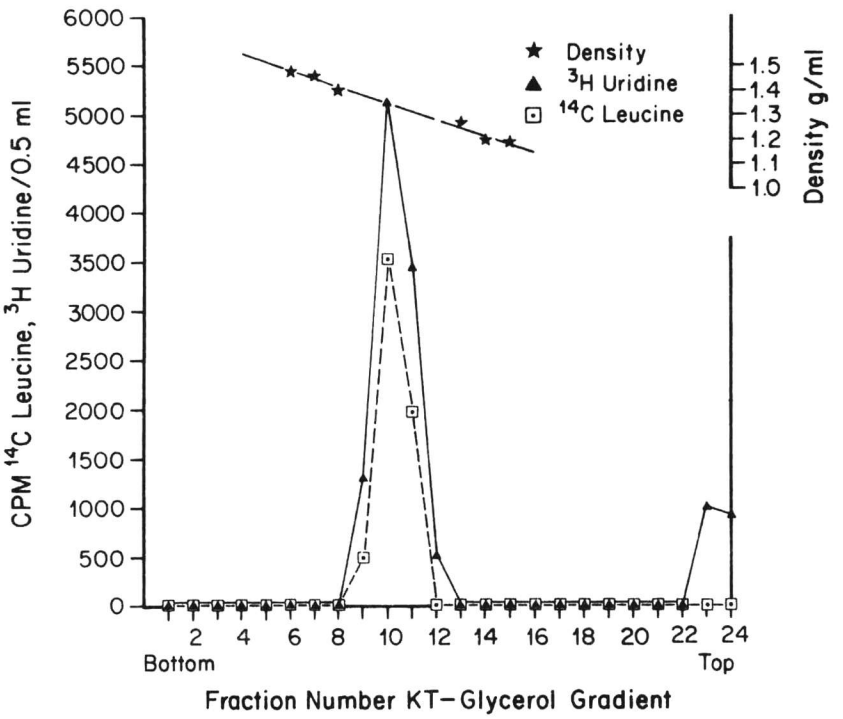

Fig. 9. Graph showing sedimentation of SA-11 rotavirus in a second KT-Glycerol equilibrium viscosity density gradient. Virus was labeled with ^{3}H uridine and ^{14}C leucine. Density measurements made as described in Materials and Methods.

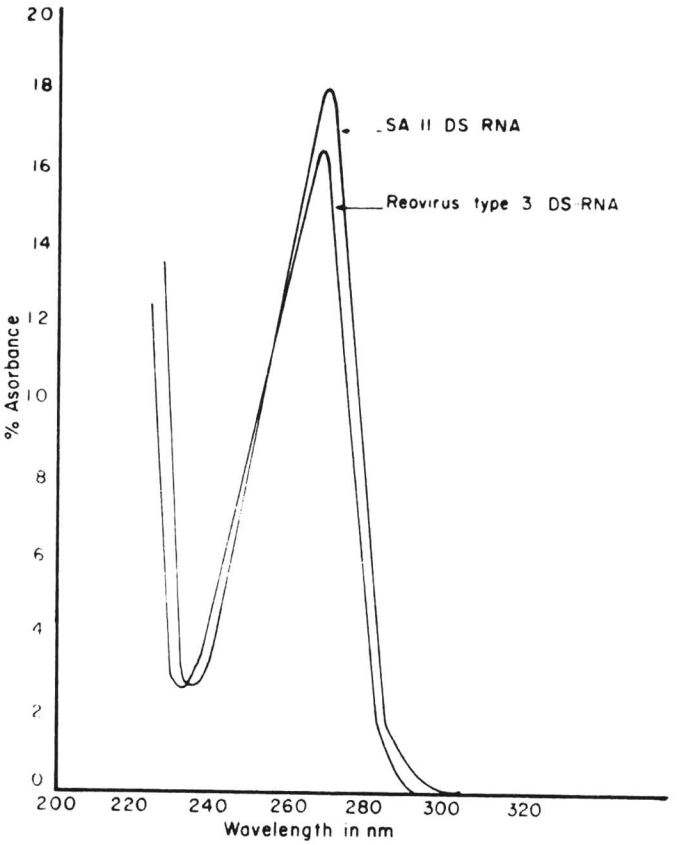

Fig. 10. Spectrophotometric scan of phenol extracted reovirus type 3 DS-RNA and SA-11 DS-RNA after alcohol precipitation and resuspension in TPE buffer.

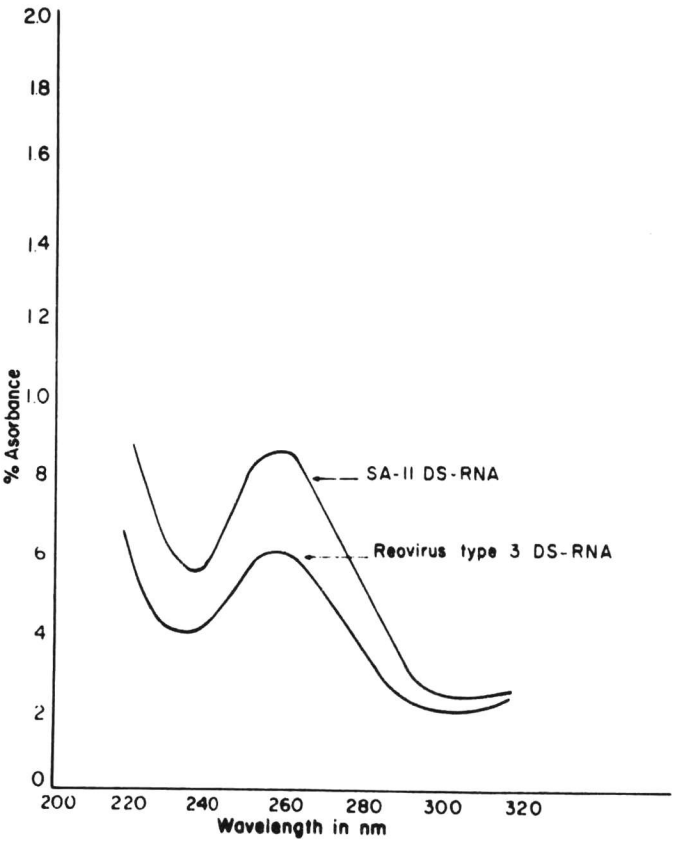

Fig. 11. Spectrophotometric scan of phenol extracted reovirus type 3 DS-RNA and SA-11 DS-RNA after dialysis against 25mM Tris-HCl and 1mM disodium EDTA.

6.2 mg of reovirus type 3 RNA are present in the samples respectively.

Detection of Glucosamine Incorporation

Virus was grown in the presence of ^{14}C glucosamine to determine if the label was incorporated into a viral glycoprotein. Following density gradient centrifugation glucosamine label was detected as a single peak at fraction number 8 (Fig. 12). Electron microscopic examination of this fraction showed it to contain intact, double-shelled particles.

Electrophoresis of Dissociated Whole Virus

The electrophoretic profiles in Fig. 13 and 14 were obtained when dissociated whole virus was electrophoresed in the disc-SDS acrylamide gel system. Nine bands were resolved. The first band was determined to be a contaminant since it did not appear in gels of labeled viral proteins nor in any subsequent experiment using different virus preparations. Molecular weight estimates were made as described in Materials and Methods and shown in Fig. 15. Estimated molecular weights for virus proteins are listed in Table 1. The molecular weights of the VSV-Indiana standard used were from Obijeski et al. (1974).

Electrophoresis of Viral DS-RNA

The electrophoretic profile in Fig. 16 was obtained when phenol extracted RNA was electrophoresed in a disc-SDS acrylamide gel system. Eleven bands were resolved. Molecular weight estimates were made as described in Materials and Methods and shown in Fig. 17. Estimated molecular weights for

Table 1. Molecular Weight Estimation of SA-11 Rotavirus Proteins

Virus Protein	Molecular weight ($x10^3$)
VP1	113
VP2	88
VP3	83
*VP4	59
VP5	38.9
VP6	34.8
VP7	32
VP8	30.6

*Not always resolved

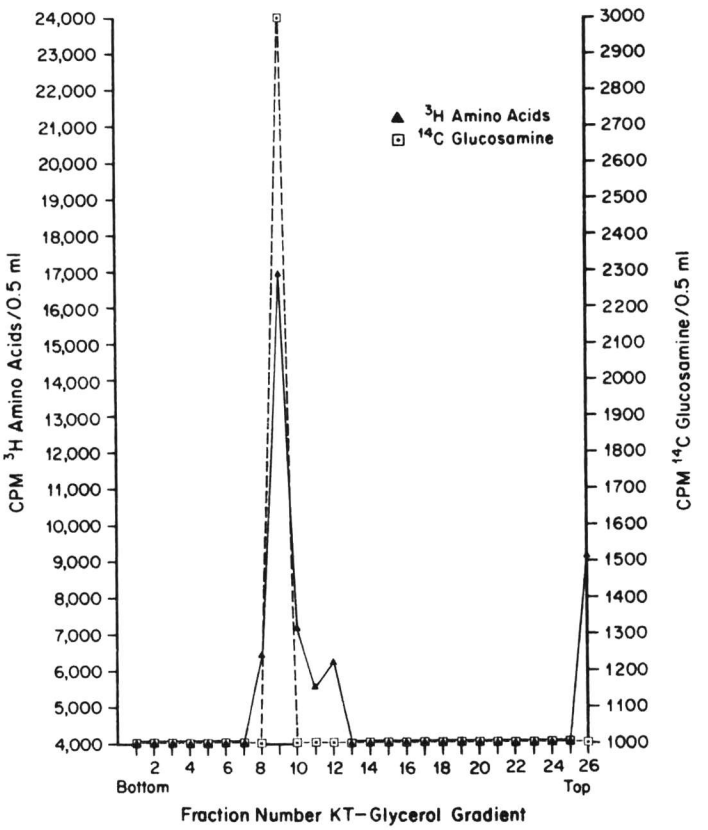

Fig. 12. Graph showing sedimentation of SA-11 rotavirus in a KT- Glycerol equilibrium density gradient. Virus was labeled with ^{3}H amino acids and ^{14}C glucosamine.

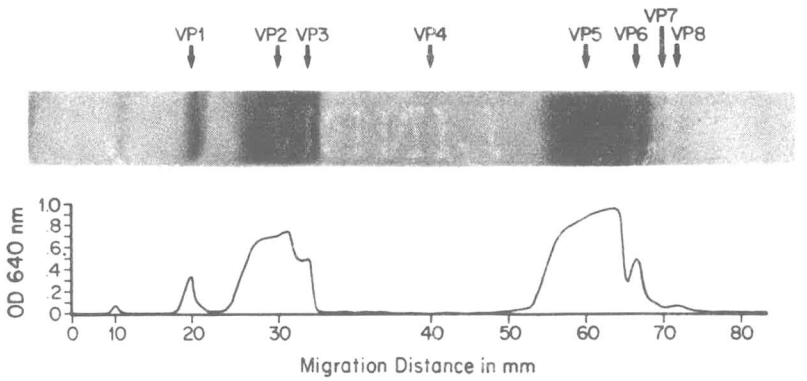

Fig. 13. Photograph showing separation of SA-11 rotavirus polypeptides on a 10% SDS-polyacrylamide disc gel (upper). Polypeptides were stained with Coomassie brilliant blue. Densitometric scan made from above gel (lower).

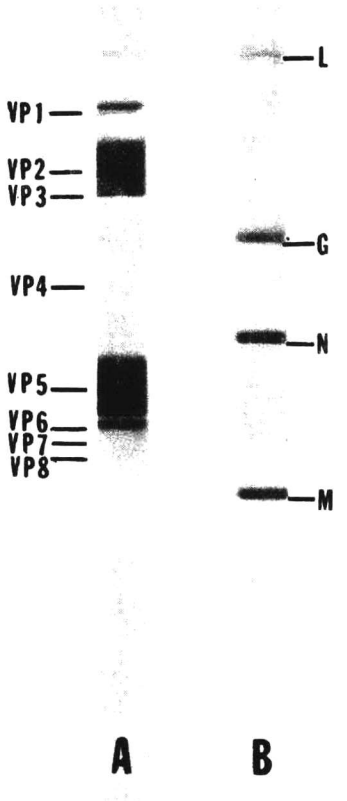

Fig. 14. Photograph showing separation of SA-11 rotavirus (A) and VSV (Indiana) polypeptides (B) in a 10% SDS-polyacrylamide-disc gel.

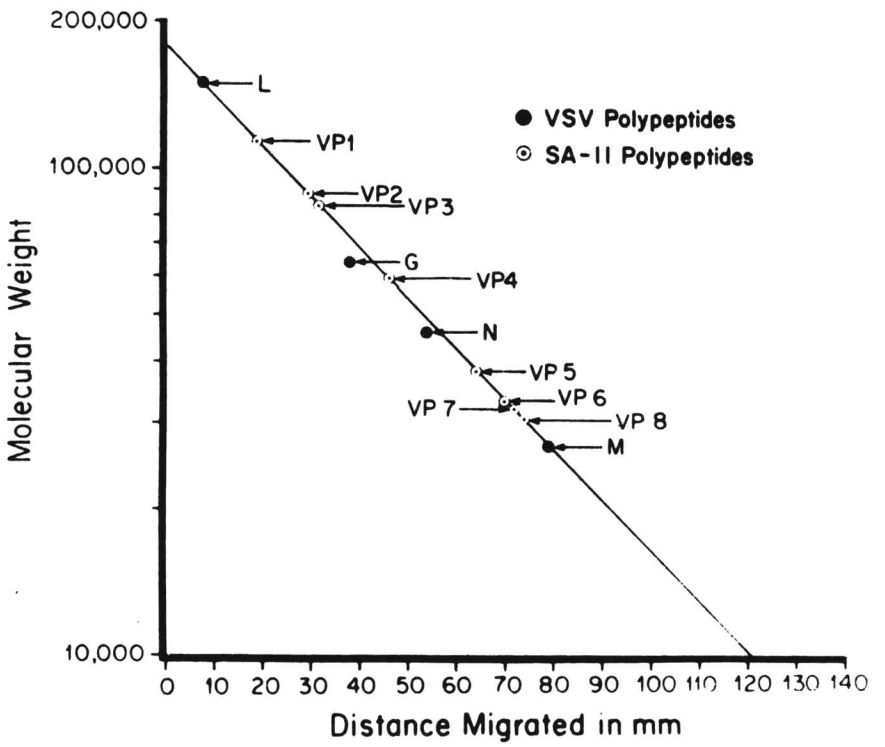

Fig. 15. Graph showing molecular weight estimates of SA-11 rotavirus polypeptides as determined by SDS-PAGE. VSV (Indiana) polypeptides were used as standards.

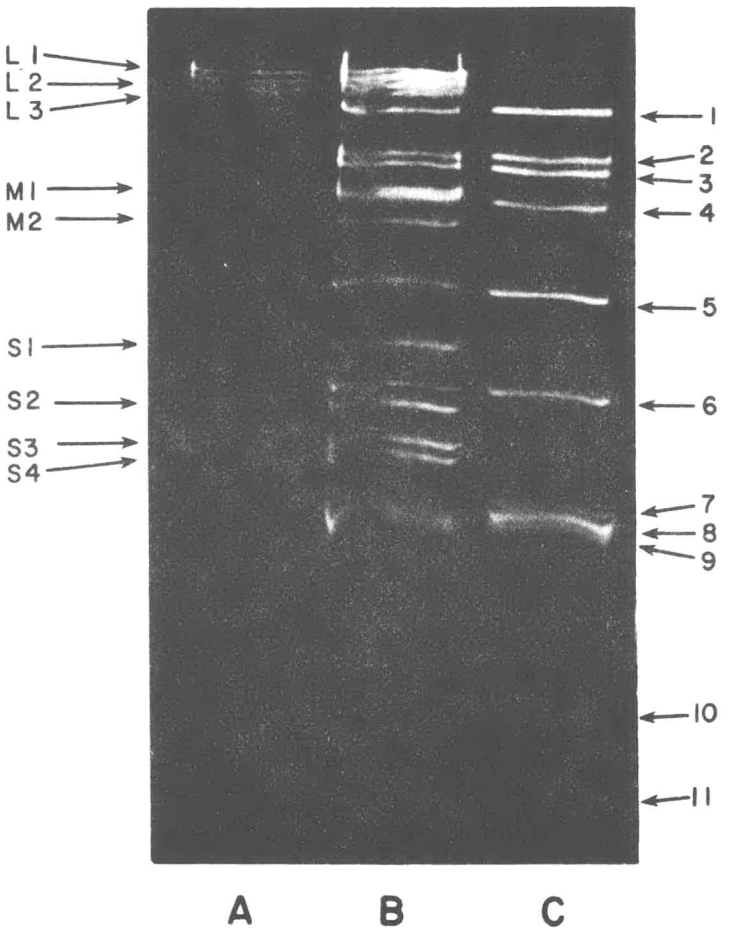

Fig. 16. Photograph showing the separation of SA-11 rotavirus DS-RNA (phenol extracted) on a 7.5% SDS-polyacrylamide- disc gel. RNA stained with EtBr included in electrode buffer. (A) Reovirus type 3 DS-RNA. (B) Co-electrophoresis of SA-11 rotavirus DS-RNA and reovirus type 3 DS-RNA. (C) SA-11 DS-RNA.

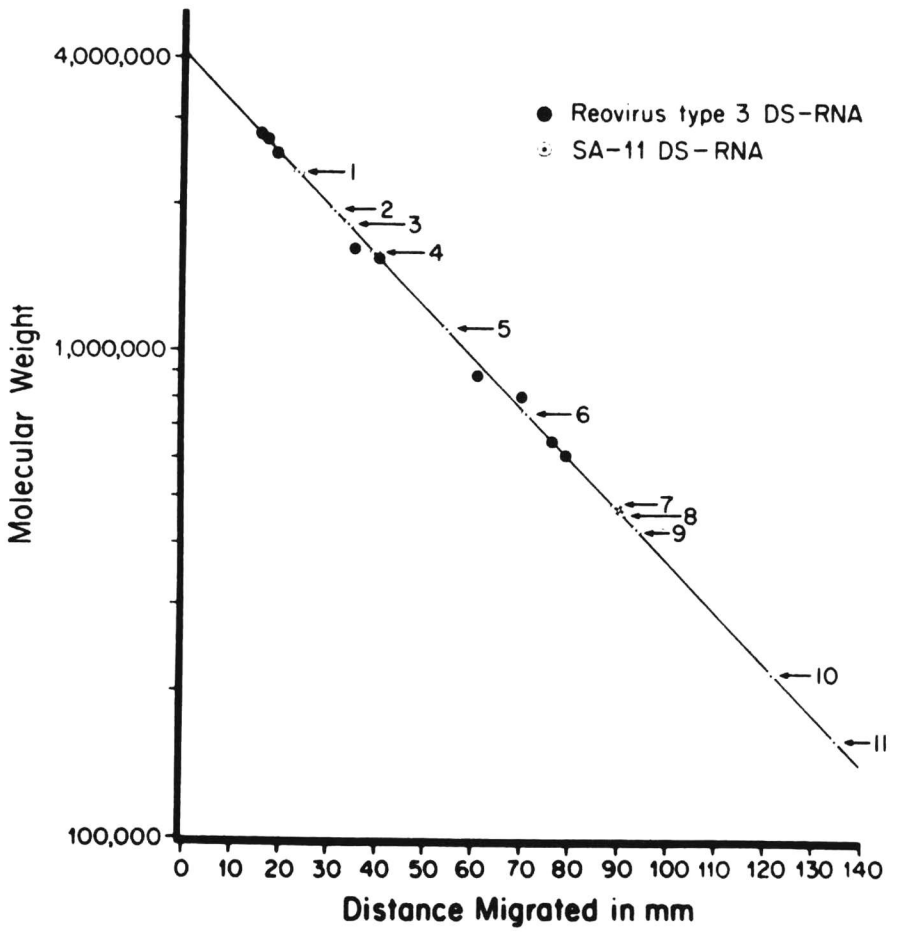

Fig. 17. Graph showing molecular weight estimates of SA-11 rotavirus DS-RNA as determined by SDS-PAGE. Reovirus type 3 DS-RNA used as standard.

virus DS-RNA are listed in Table 2. The molecular weights of the Reovirus type 3 DS-RNA standard used were from Martin and Zweerink (1972).

Fluorography

Following electrophoresis dissociated whole labeled virus acrylamide gels were fluorographed as described in Materials and Methods. An early fluorograph (not shown) indicated that both radioisotope labeled RNA and proteins were present on the same gel. This led to an experiment to determine if RNA and protein could be resolved on the same gel by the staining procedures described in Materials and Methods and later fluorographed.

Dissociated whole virus was electrophoresed in an electrophoretic system which included EtBr in the electrode buffer. In this way the migration of RNA segments was followed by exposing the gel to UV light. After electrophoresis this gel was photographed under UV light for RNA (Fig. 18), stained for protein, destained and photographed (Fig. 19). The same gel was fluorographed yielding the pattern seen in Fig. 20. This RNA profile is the same as in Fig. 17 and the protein pattern is the same as in Fig. 18. The RNA pattern resolved on both the fluorograph and EtBr staining are similar to the pattern in Fig. 16. However, there are three bands shown in Fig. 17 and 18 which are absent from Fig. 16. Although molecular weight estimations were not made as described in Materials and Methods, it is easy to see that their molecular weights are between 1.15×10^6 and 1.57×10^6.

Table 2. Molecular Weight Estimates of SA-11 Rotavirus RNA

Size Class	Segment Number	Molecular Weight ($x10^6$)
I	1	2.41
	2	1.93
	3	1.82
	4	1.57
II	5	1.15
	6	0.790
III	7	0.475
	8	0.470
	9	0.432
IV	10	0.220
	11	0.160
Total	11	11.427

Fig. 18. Photograph showing the separation of SA-11 rotavirus DS-RNA (from dissociated whole virus, not phenol extracted) on a 8% polyacrylamide disc gel. RNA stained with EtBr included in electrode buffer.

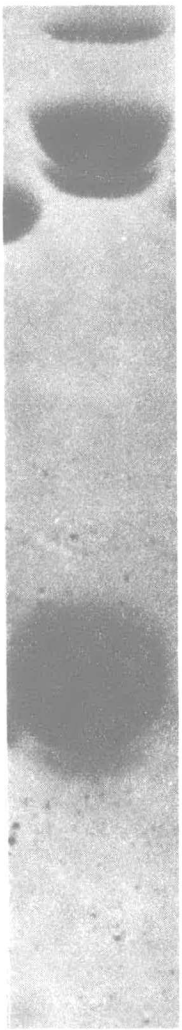

Fig. 19. Photograph showing the separation of SA-11 rotavirus polypeptides on a 8% polyacrylamide disc gel. Stained with Coomassie brilliant blue.

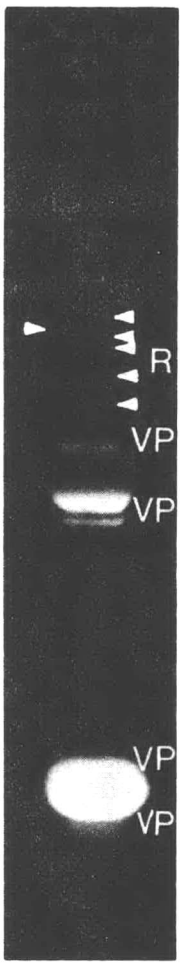

Fig. 20. Fluorograph of a 8% polyacrylamide disc gel. RNA (R) labeled with ^{3}H uridine and protein (VP) labeled with ^{14}C leucine.

CHAPTER V

DISCUSSION

The infectivity of some species of rotavirus has been shown to be enhanced by including trypsin in the growth medium (Babiuk et al., 1977, Matsuno et al., 1977, Almeida et al., 1978; Matsuno et al., 1979) or by pretreating innoculum with trypsin (Clark et al., 1979). In this study, SA-11 rotavirus infectivity was also found to be enhanced by trypsin. Trypsin treated virus produced gross CPE 18-24 hrs post-infection. Conversely, virus not treated with trypsin prior to innoculation took 3-4 days to become overtly cytopathic. The mechanism involved in the enhancement of infectivity by trypsin is not known. Babiuk et al. (1977) and Almeida et al. (1978) suggest that rotaviruses are good inducers of interferon and that these viruses are inactivated by the action of interferon. Their theory is based on results of Issacs (1957) which show that trypsin does, in fact, degrade interferon. A fibro-epithelial interferon is known to be produced by fibroblast and epithelial cells (Baron, 1979).

A possible mechanism explaining enhanced infectivity of rotaviruses by trypsin suggested by Clark et al. (1979) is that trypsin increases infectivity by dispersing viral aggregates. Another possible mechanism offered by this investigator is that trypsin digests portions of viral polypeptides which partially mask virus attachment sites. Alleviating the masking effect

enhances virus attachment and ultimately virus replication. It is also possible that both of the immediately preceding mechanisms might be responsible.

In this study when distilled water, physiological saline or solutions of tris-base were used as the solvent for the preparation of gradients and buffers, virus integrity was found to be decreased. However Ca^{+2} containing TCN buffer enhanced maintenance of virus integrity is in agreement with results of Cohen, et al. (1979) who noted that Ca^{+2} ions stabilized calf rotavirus. Taken together these data support the hypothesis of Durham (1977) that calcium ions play a crucial role in controlling the disassembly of some icosahedral viruses.

Cohen (1977) reported that calf rotavirus contained glycosylated proteins and proposed that the terminal residue was mannose. In this study ^{14}C glucosamine was used to determine if SA-11 rotavirus possessed any glucosamine residues. It was shown that the fractions from equilibrium viscosity density gradients having a peak of coincidence label of ^{14}C glucosamine and ^{3}H amino acids contained complete, double-shelled particles. Matsuno and Mukoyama (1979) reported that glucosamine was associated with a viral protein of molecular weight 2.8×10^4 in calf rotavirus. Rodger et al. (1977) reported that a 2.3×10^4 molecular weight protein of SA-11 rotavirus was glycosylated (by PAS stain). A detailed study of the glycosylated rotavirus residues is being conducted by this investigator.

Eight proteins were resolved on SDS-PAGE analysis of dissociated SA-11 rotavirus double-shelled virions. The estimated

molecular weights ranged from 3.06×10^4 to 1.13×10^4. Two major proteins (VP2, MW 8.8×10^4 and VP5, MW 3.89×10^4) and 6 minor proteins were resolved. A 5.9×10^4 molecular weight protein was not always resolved. These findings are similar to reports by Kalica and Theodore (1979) and Rodger et al. (1977) who reported resolving 8 and 9 proteins respectively. The pattern resolved here is very similar to one reported by Rodger et al. (1977). However, the high molecular weight polypeptides for SA-11 rotavirus were found to be lower than those reported by Rodger et al. (1977) or Kalica and Theodore et al. (1979) and the lower range molecular weight estimates are also lower than the values (4.8×10^4 - 8.7×10^4) of Kalica and Theodore (1979). It should be noted that the molecular weight estimates of Kalica and Theodore (1979) are not in agreement with values for other rotavirus polypeptides. With the exception of VP1, the remainder of their molecular weight estimates are considerably different from those reported by others. Furthermore, the electrophoretic pattern of the viral structural polypeptides in their report is not similar to other reports. They assign a 6.7×10^4 molecular weight polypeptide (VP7 from their report) to the outer capsid. This polypeptide has been designated as an inner coat protein by investigators (Rodger et al., 1975, Rodger et al., 1977, Todd and McNulty, 1977, Matsuno et al., 1979 and Thouless, 1979).

Because of these inconsistencies this investigator believes that the report by Rodger et al. (1977) presents a better model

model for comparison. The differences in the molecular weight estimates of SA-11 rotavirus reported here and those of Rodger et al. (1977) may be because the virus employed in this study has been passaged in tissue culture numerous times and these variations might well reflect some alteration of the virions structural polypeptides on an evolutionary line, or since the pattern for SA-11 rotavirus reported here resembles that of Rodger et al. (1977) the differences in estimated molecular weights may be the result of variation in gel systems used and/or construction of standard plot.

VP4, VP7 and VP8 are believed to be outer-shell components since in preparations having many single-shelled particles or preparations which were not very concentrated these polypeptides appeared faintly if at all. By subtraction then VP1, VP2, VP3, VP5 and VP6 are believed to be inner shell components. It should be noted that conditions yielding high titers of complete, double-shelled SA-11 particles in tissue culture are just being developed. Once optimal growth conditions have been defined pulse labelling experiments should provide more definite answers to the questions of number and location of the viral polypeptides.

Eleven bands were resolved from phenol extracted samples of SA-11 rotavirus RNA preparations. The electrophoretic pattern and molecular weight estimates are similar to those of other rotaviruses (Rodger et al., 1975, Kalica et al., 1976, Todd and McNulty, 1977 and Wyatt et al., 1978). The estimated

molecular weight of the viral genome is 11.43 x 10^6 with a range of 1.6 x 10^5 to 2.41 x 10^6.

Kalica et al. (1976) described 4 size classes for rotavirus RNA based partly on contour lengths. The eleven segments reported here could be divided into similar size classes. Segments 1-4 comprise class I, 5 and 6 class II, 7, 8 and 9 class III and segments 10 and 11 class IV. These molecular weight estimates described here are the first reported for SA-11.

SDS-PAGE analysis of RNA from DDB dissociated virions but not phenol extracted revealed 3 additional bands. These bands appear between segments labeled 4 and 5 of phenol extracted preparations and have molecular weights estimated between 1.15 x 10^6 and 1.57 x 10^6. These segments represent RNA having small amounts of associated protein. This would mean that they are in reality segments 5 and 6 but owing to the associated protein their migration patterns are different. It would be interesting to perform in vitro polypeptide synthesis using these segments and comparing the nascent polypeptides with counterparts resulting from transcription and translation of segments 5 and 6. There is a also a possibility that these species represent RNA trapped in the interphase during phenol extraction. They could represent RNA species responsible for additional polypeptides, i.e., enzymes required for viral synthesis. These polypeptides may be short lived and are therefore not resolved upon subsequent analysis of mature virions.

Fluorography of labeled virus yielded patterns for proteins and RNA similar to those obtained from Coomassie brilliant blue and EtBR staining. However VP4 (MW 5.9 x 10^4) was not resolved and all RNA species were not apparent. Those RNA segments not apparent were obscured by larger protein bands in the region. The question of how viral proteins having much lower molecular weights could band in regions with RNA of much higher molecular weights might arise. A possible explanation for this is the fact that even though both RNA and protein were resolved on the same gel their migration within the electric field need not be the same owing to spatial differences of the protein and double-stranded RNA.

CHAPTER VI

SUMMARY

The purpose of this study was to characterize the structural polypeptides and RNA of simian rotavirus SA-11. Virus was propogated in MA-104 (embryonic rhesus) monkey kidney cells. Viral polypeptides and RNA were analysed by SDS-PAGE.

It was determined that:

(1) Pre-treatment of SA-11 rotavirus innoculum with trypsin greatly enhanced virus infectivity.

(2) Maintenance of viral integrity was shown to require the presence of calcium ions.

(3) Equilibrium viscosity density gradient sedimentation of complete, double-shelled virions in KT-Glycerol indicated that the virions had a density of 1.35g/ml.

(4) Eight polypeptides were resolved from complete, double-shelled particles. The estimated molecular weight range was $3.06 \times 10^4 - 1.13 \times 10^5$. Two major polypeptides were resolved which are believed to be associated with the inner capsid of the virus. VP1, VP2, VP3, VP5 and VP6 are probably inner capsid polypeptides while VP4, VP7 and VP8 are probably outer capsid polypeptides. One or more of these polypeptides are probably glycosylated having some glucosamine residues.

(5) Analysis of phenol extracted RNA indicated the presence of 11 segments which could be divided into 4 size classes.

The molecular weight range was 1.6×10^5 - 2.41×10^6. Class I was composed of segments 1, 2, 3 and 4, class II segments 5 and 6, class III segments 7, 8 and 9 and class IV segments 10 and 11. The migration of RNA segments could be followed by exposing gels to UV light.

(6) RNA and protein could be resolved on a single gel by staining (EtBr and Coomassie brilliant blue) and fluorography. If future work confirms the validity of results obtained from such a procedure the amount of sample required, time required for analysis and cost of materials will be reduced significantly.

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